Journal of Chromatography, 505 (1990) 329–347
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 323

Two-component protein adsorption to the cation exchanger S Sepharose® FF

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(First received September 7th, 1989; revised manuscript received February 2nd, 1990)

SUMMARY

A model system, consisting of bovine serum albumin, lysozyme and the cation exchanger S Sepharose® FF, was used to investigate multicomponent protein adsorption to ion exchangers. Two models, one based on a complete absence of competition between adsorbing molecules and the other a competitive model, based on the assumption that all adsorption sites are available to both proteins, have been compared to experimental results. Evidence for competitive adsorption was seen in experiments in which breakthrough curves and the profiles of adsorbed proteins in packed beds were determined. However, although the results for packed-bed experiments were more closely predicted by the fully competitive model, some discrepancies were found suggesting that when considering multicomponent protein adsorption to ion exchangers it may also be necessary to take account of factors such as the molecular size of the adsorbing proteins and any potential inter-protein interactions, factors which may hinder the development of a general model of multicomponent protein adsorption to ion exchangers.

INTRODUCTION

Capital expenditure on both process equipment and consumable materials can account for significant proportions of the costs of large scale production of pharmaceutical proteins. It is desirable therefore, to design and optimise the purification protocol to keep this expenditure to a minimum. The design of a purification process requires the use of a model which is able to predict the required parameters. Whilst there are many published studies of the adsorption of single proteins to different adsorbents, few studies have been reported using more realistic multicomponent systems which involved more than one adsorbing protein, a fact that has been pointed out by Janson and Hedman[1]. A theoretical discussion of an approach to modelling multicomponent protein adsorption has been published by Velayudhan and Horváth[2], although they did not present any associated experimental data. Experimental results from a non-protein system have been published in an earlier

0021-9673/90/$03.50

paper from the same laboratory[3]. This paper presented two methods for determining competitive adsorption isotherms from the results of frontal analysis chromatography and included a theoretical discussion of the derivation and use of the parameters of the Langmuir isotherm. Results of studies of the adsorption of a mixture of albumin and β-lactamase to encapsulated ion exchangers have been published recently by Nigam *et al.*[4]. They report that competition between the two adsorbing proteins occurred, with albumin displacing the more weakly binding β-lactamase from the ion exchanger.

In order to commence studying multicomponent adsorption, we have examined a model system consisting of two adsorbing species, bovine serum albumin (BSA) and lysozyme, and the strong cation exchanger S Sepharose® FF. We have studied the characteristics of the adsorption of each pure protein to S Sepharose FF and the results of those studies were fully reported in a previous paper[5]. Those studies showed that the Langmuir adsorption isotherm could be used to describe the equilibrium adsorption characteristics of both proteins. In addition, using parameters from the Langmuir isotherm, two different models of the rate of uptake of protein, a kinetic rate constant model and a film and pore diffusion model, were presented. The predictions for the adsorption of lysozyme to S Sepharose FF in both batch, agitated tanks and packed beds were in close agreement with the experimental results. However in the case of BSA, only the film and pore diffusion model was in close agreement with the actual adsorption profile seen in batch, agitated tanks, whilst neither model agreed precisely with the results obtained in column experiments. This was largely due to the fact that the observed breakthrough curves are asymmetric and approach the inlet concentration very slowly. This effect is more pronounced in experiments with BSA, but is observed nevertheless in several different protein–adsorbent systems and makes calculation of the amount of protein adsorbed at equilibrium difficult in these packed-bed experiments.

In this paper we present the results of studies of multicomponent adsorption of BSA and lysozyme to S Sepharose FF and compare these to two models, one based on competitive adsorption to the ion exchanger and the other based on non-competitive adsorption. The choice of proteins for these studies was determined on two grounds. Firstly the high capacity of ion exchangers for proteins meant that gram quantities of pure proteins were required at reasonable cost. Secondly there had to be a method of quantifying the amounts of each protein present in a mixture. The difference in molecular size between BSA and lysozyme enabled the proteins to be separated by molecular-exclusion chromatography and the amounts of the two proteins could be determined by analysis of the resulting chromatogram.

THEORY

Analysis of multicomponent adsorption to ion exchangers benefits from an understanding of the nature of the protein adsorption sites on the adsorbent. Proteins adsorb to ion exchangers as a result of ionic interactions between charged groups on the surface of the protein and oppositely charged groups on the ion exchanger. A protein molecule carries many charged groups and multiple ionic interactions will occur with the adsorbent. Gosling[6] for example, has demonstrated the expulsion of 15–20 chloride counter ions from a diethylaminoethyl-exchanger for each mole of BSA adsorbed. As the three-dimensional distribution of ionic groups on the surface of the

adsorbent is random, the actual protein adsorption site is not a unique entity. Hence the adsorption site on a protein ion exchanger cannot strictly be treated in the same manner as that postulated for affinity adsorption where molecules of the immobilised affinity ligand constitute adsorption sites with identical properties. A Langmuir type isotherm might not be expected to describe the adsorption of single proteins to ion exchangers as fundamental thermodynamic conditions such as the identical nature of adsorption sites and an absence of lateral interaction between adsorbed solute molecules are not obeyed. However, experimental results from systems in which a single protein is adsorbed to an ion exchanger yield equilibrium isotherms which can be described by a Langmuir equation of the form shown below, for example see Leaver[7], Annesini and Lavecchia[8] and Graham *et al.*[9]:

$$q^* = \frac{c^* q_m}{c^* + K_d} \tag{1}$$

where q represents the concentration of protein adsorbed to the ion exchanger, q_m represents the maximum concentration of protein that can be adsorbed to the ion exchanger, c the concentration of protein in the bulk solution and K_d is the dissociation constant of the protein–ion exchanger complex. The superscript * indicates equilibrium values.

In particular, our previous experiments[5] involving the single-component adsorption of BSA and lysozyme to S Sepharose FF show that the equilibrium adsorption isotherms are of the above form and the equilibrium adsorption parameters determined in those studies are presented in Table I. The observation of a Langmuir-type shape can probably be explained by protein adsorption to the ion exchanger continuing until there is no longer room on the surface of the adsorbent for further molecules of adsorbate to bind. Hence further adsorption ceases once monolayer coverage has occurred. This eventual saturation of the adsorbent surface leads to adsorption isotherms of a similar shape to the Langmuir isotherm even though the underlying thermodynamic assumptions are not strictly obeyed, and the Langmuir isotherm therefore is widely used as a simple empirical model of the equilibrium adsorption characteristics of various protein–adsorbent systems.

In addition to uncertainties arising from the undefined nature of the adsorption sites on an ion exchanger for the adsorption of a single, pure protein, the situation is further complicated when the adsorption of two or more proteins is being considered. As a result of the different sizes and distribution of charges on the surfaces of different

TABLE I

VALUES OF K_d AND q_m FOR THE ADSORPTION OF PURE BSA AND LYSOZYME TO S SEPHAROSE FF

The values were determined as described previously[5].

	K_d (mg ml^{-1})	K_d (M)	q_m (mg ml^{-1})	q_m (mol l^{-1})
BSA	0.133	$2.0 \cdot 10^{-6}$	113	$1.7 \cdot 10^{-3}$
Lysozyme	0.019	$1.3 \cdot 10^{-6}$	120	$8.4 \cdot 10^{-3}$

proteins, the number of ionic groups that will participate in the adsorption interaction and the amount of adsorbent surface which interacts with the different proteins will vary. Whilst recognising the complexities of multicomponent adsorption of proteins to ion exchangers, in this paper we have adopted two extreme views to analyse two-component protein adsorption, namely a non-competitive model and a totally competitive model. Both of these models are based on the Langmuir adsorption isotherm, an approach supported by our earlier single-component studies of the adsorption of BSA and lysozyme to S Sepharose FF[5]. It is shown that the totally competitive model more accurately describes the experimental observations.

Non-competitive adsorption model

One extreme view of the adsorption of two proteins to an ion exchanger is to assume that the adsorption sites for the two proteins are mutually independent, that is the adsorption of one type of protein to the ion exchanger in no way affects the adsorption of the other species and there is therefore no competition between the proteins for the adsorption sites. This may be an unlikely situation as the fundamental mechanism for the adsorption of both types of protein molecules involves interaction with the available ionic groups on the surface of the exchanger. If there is no competition between the proteins for adsorption, the adsorption characteristics of each protein will be the same as if the other protein were not present, *i.e.*

$$q_1^* = \frac{c_1^* q_{m1}}{c_1^* + K_{d1}} \quad \text{and} \quad q_2^* = \frac{c_2^* q_{m2}}{c_2^* + K_{d2}} \tag{2}$$

where the subscripts 1 and 2 indicate adsorbate species 1 and 2.

Totally competitive adsorption model

The other extreme approach to the analysis of two-component adsorption is to assume that there is total competition between proteins for adsorption to the ion exchanger. Although the exchanger shows different maximum capacities for the two proteins (q_{m1} and q_{m2}), a competitive model can be developed which involves a fractional occupancy of the adsorption capacity for each type of protein and uses Langmuir parameters derived from single-component experiments. It should always be borne in mind that such an approach violates the Gibbs–Duhem relationship (see ref. 10) and that a thermodynamically rigorous approach would demand the use of parameters determined from two-component experiments. However in the present study it was assumed that at the micromolar concentrations of soluble protein employed in these experiments, the use of parameters determined in single-component studies was a sufficiently accurate approximation.

Let θ represent the fractional occupancy of the adsorbent at equilibrium with a particular protein, such that:

$$\theta_1 = \frac{q_1^*}{q_{m1}}; \qquad \theta_2 = \frac{q_2^*}{q_{m2}} \tag{3}$$

The fraction of unoccupied sites is therefore given by $(1 - \theta_1 - \theta_2)$. At equilibrium

$$K_{d1} = \frac{c_1^*(1 - \theta_1 - \theta_2)}{\theta_1}; \qquad K_{d2} = \frac{c_2^*(1 - \theta_1 - \theta_2)}{\theta_2} \tag{4}$$

giving

$$c_1^* = \theta_1(K_{d1} + c_1^*) + c_1^*\theta_2; \qquad c_2^* = \theta_2(K_{d2} + c_2^*) + c_2^*\theta_1 \tag{5}$$

and

$$(1 - \theta_1 - \theta_2) = \frac{K_{d1}\theta_1}{c_1^*} = \frac{K_{d2}\theta_2}{c_2^*} \tag{6}$$

From eqn. 6:

$$c_1^*\theta_2 = \frac{K_{d1}}{K_{d2}} c_2^*\theta_1 \tag{7}$$

So substituting into eqn. 5 for $c_1^*\theta_2$ from eqn. 7 and for θ_1 from eqn. 3 gives:

$$q_1^* = \frac{q_{m1}c_1^*}{K_{d1} + c_1^* + \dfrac{K_{d1}}{K_{d2}}c_2^*} \tag{8}$$

and similarly

$$q_2^* = \frac{q_{m2}c_2^*}{K_{d2} + c_2^* + \dfrac{K_{d2}}{K_{d1}}c_1^*} \tag{9}$$

The equilibrium position of a batch system can be determined by solving eqns. 8 and 9 simultaneously with the mass balance equations:

$$Vc_{01} = Vc_1^* + vq_1^* \tag{10}$$

$$Vc_{02} = Vc_2^* + vq_2^* \tag{11}$$

where V is the volume of the liquid phase and v is the settled volume of adsorbent in the system. c_{01} and c_{02} are the initial concentrations of the two proteins. A program was written in BASIC to solve eqns. 8, 9, 10 and 11 for values of c_i^* and q_i^* for a particular set of initial conditions V, v and c_{0i} by an iterative method using the values of K_{di} and q_{mi} determined in single-component adsorption isotherm measurements.

EXPERIMENTAL

Materials

BSA and lysozyme (EC 3.2.1.17) were obtained from Sigma (Poole, U.K.), catalogue Nos. A-3912 and L-6876 respectively. BSA has a relative molecular mass of 66 300 daltons[11] and an isoelectric point (p*I*) of 4.7 (ref. 12), whilst lysozyme has a relative molecular mass of 14 500 daltons[13] and a p*I* of 11.1 (ref. 14).

All solutions were buffered with 0.1 *M* sodium acetate–acetic acid, pH 5. Sodium acetate, acetic acid and sodium chloride were all laboratory-grade reagents. S Sepharose FF was a gift from Pharmacia-LKB (Uppsala, Sweden). Known volumes of ion exchanger were obtained by allowing a suspension of the ion exchanger to settle in a measuring cylinder overnight and then adjusting the liquid volume to equal that of the settled ion exchanger. Aliquots of a known volume of a 50:50 (v/v) suspension were then obtained by the use of a Gilson Pipettman automatic pipette.

Determination of protein concentration in the liquid phase

In experiments in which only one protein was present in solution, it was possible to determine protein concentration by measuring the optical density at 280 nm which could be converted to concentration by reference to calibration data. In experiments in which both BSA and lysozyme were present in solution together, quantitation of the concentrations of the individual proteins was achieved by analytical separation of the proteins by molecular-exclusion chromatography using a Fast Protein Liquid Chromatography system (FPLC™) (Pharmacia-LKB). All samples and buffer solutions for FPLC were filtered through 0.22-μm low protein binding Durapore® filters (Millipore, Harrow, U.K.). Protein samples of 200 μl were applied to an HR10/30 column of Superose™ 12 equilibrated with 0.1 *M* sodium acetate–acetic acid, pH 5. The column was eluted with this buffer at a flow-rate of 1 ml/min. This flow-rate, higher than usually recommended for good performance using this column, still resulted in resolution of the two proteins due to the large difference in molecular weight between BSA and lysozyme and allowed a sample to be analysed in 25 min. Integration of the peaks on the resultant chromatogram was performed by the LCC-500 chromatography controller unit of the FPLC system. The concentrations of each protein were then determined from the areas of their peaks by reference to calibration data.

The FPLC method for determining protein concentration was validated by comparing the protein concentration in the outlet stream from packed beds loaded with a single pure protein, determined directly from optical density measurements at 280 nm, with the concentrations determined using the FPLC method. The results of the two determinations were in close agreement and it was decided that the concentration of each protein in samples from the two-component experiments could be accurately determined solely from FPLC analysis. It was also established that the presence of salt had no effect on the separation by FPLC of the two proteins, nor on the quantitation of the integrated areas, thus samples eluted from S Sepharose FF in 1 *M* sodium chloride could be analysed for protein composition with no pretreatment.

Batch equilibrium adsorption studies

A number of experiments were performed in which samples of S Sepharose FF

were equilibrated with different mixtures of BSA and lysozyme. The experiments were performed in flasks prepared according to the protocol for determining adsorption isotherms described previously[5], with the difference that each flask contained not one protein but a mixture of the two proteins. The total mass of protein present in each flask was varied whilst the volume of solution and S Sepharose FF was kept constant. The amounts of BSA and lysozyme used in each flask were always equal on a mass basis. The flasks were incubated overnight in a shaking water bath at 25°C to allow equilibrium to be established. At equilibrium the amount of each protein present in the liquid phase was determined by FPLC as described above, allowing the amounts of each protein that were adsorbed to the ion exchanger to be calculated by mass balance.

Packed-bed experiments

All column experiments were performed with 2 ml (settled volume) of S Sepharose FF packed in a chromatography column, 1 cm diameter (0.785 cm^2 cross-sectional area), mounted vertically. It was found that the volume of ion exchanger used gave a bed height of 2.2–2.3 cm, equivalent to a packed volume of approximately 1.75 ml. All experiments were performed at a volumetric flow-rate of 1 ml/min (superficial velocity 1.27 cm/min) and flow was always in an upward direction. Optical density at 280 nm of the outlet stream was recorded and fractions of 2 ml were collected at the column exit as required for FPLC analysis as described above.

Determination of adsorbed protein profiles in packed beds

A series of packed-bed experiments, in which the bed was loaded with a two-component protein mixture for 50, 100, 150, 200, 250 and 400 min, was performed. At the end of the loading period liquid was removed from the bed by passing 5 ml of air through the bed with a syringe in the reverse direction to that of the original liquid flow. The column was then clamped and the upper endpiece removed. The ion-exchange bed was then extracted from the column by pushing the lower endpiece upwards with a threaded rod. As the adsorbent emerged from the column, slices of approximately 2–3 mm (giving 9–12 slices in total from each bed) were removed with a scalpel and placed into weighed bijou bottles. The bottles were sealed and reweighed. The amount of ion exchanger present in each slice was calculated from these weights. The adsorbed proteins were eluted from the ion exchanger in each sample with 5 ml of 1 *M* sodium chloride. The bottles were agitated for approximately 15 min, at which point the S Sepharose FF was allowed to settle under gravity and 4 ml of supernatant removed. The concentrations of BSA and lysozyme present in this solution were then determined by FPLC. The amount of each protein that had been adsorbed on each slice of S Sepharose FF could then be calculated.

Consecutive application of single-protein solutions to packed beds

Packed-bed experiments, in which a feed solution containing only one of the proteins was applied, were performed. When the protein concentration of the outlet stream (c), as determined from optical density measurements equaled, or was approaching, that of the inlet stream (c_0), the incoming feed stream was switched to a solution containing only the other protein. Fractions were collected at the column exit for analysis by FPLC.

RESULTS

Batch equilibrium adsorption studies

The results of the batch equilibrium adsorption experiments are plotted in Fig. 1. From each adsorption experiment a pair of equilibrium adsorption results was obtained, one for each of the proteins present. Each result represents the concentration of protein in solution that was in equilibrium with an adsorbed amount of the same protein. When studying Fig. 1 it is important to remember that from each flask a result was obtained for each protein, therefore each point for BSA, in the order of increasing soluble protein concentration, pairs with a corresponding lysozyme point, also in order of increasing soluble protein concentration.

The experimental data are compared to the results predicted by the two models of two-component adsorption. The values of q_{mi} and K_{di} used in the predictions were those determined previously[5] in single-component experiments and presented in Table I. The non-competitive model (Fig. 1a) gave a fairly accurate prediction of the lysozyme adsorption results but greatly overpredicted the amount of BSA that would be adsorbed to the ion exchanger. Conversely the totally competitive model (Fig. 1b) gave a good prediction of the amount of BSA adsorbed but underpredicted the amount of lysozyme adsorbed.

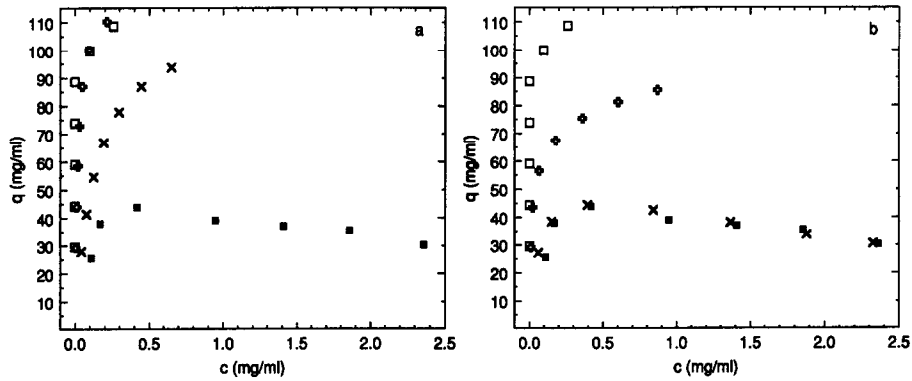

Fig. 1. Batch adsorption of a mixture of lysozyme and BSA to S Sepharose FF in 0.1 *M* acetate buffer, pH 5 at 25°C. The boxes represent experimental results for lysozyme (□) and BSA (■). The crosses represent the predicted values for lysozyme (+) and BSA (×). The data are plotted as mg protein adsorbed per ml S Sepharose FF (q) against mg/ml protein in solution (c). Each BSA point in the order of increasing soluble protein concentration pairs with a lysozyme point also in the order of increasing soluble protein concentration. (a) The experimental results are plotted with the results calculated by the non-competitive model. (b) The experimental results are plotted with the results calculated by the fully competitive model.

Frontal analysis

The development of the breakthrough profiles for BSA and lysozyme when a solution containing a mixture of each protein at a concentration of 1 mg/ml was passed through a bed of S Sepharose FF are shown in Fig. 2a. This figure shows that the breakthrough of BSA occurs before that of lysozyme, with the breakthrough profiles of both proteins having similar slopes. The concentration of BSA is seen to rise above that of the inlet concentration ($c/c_0 > 1$) before it falls back towards it. This

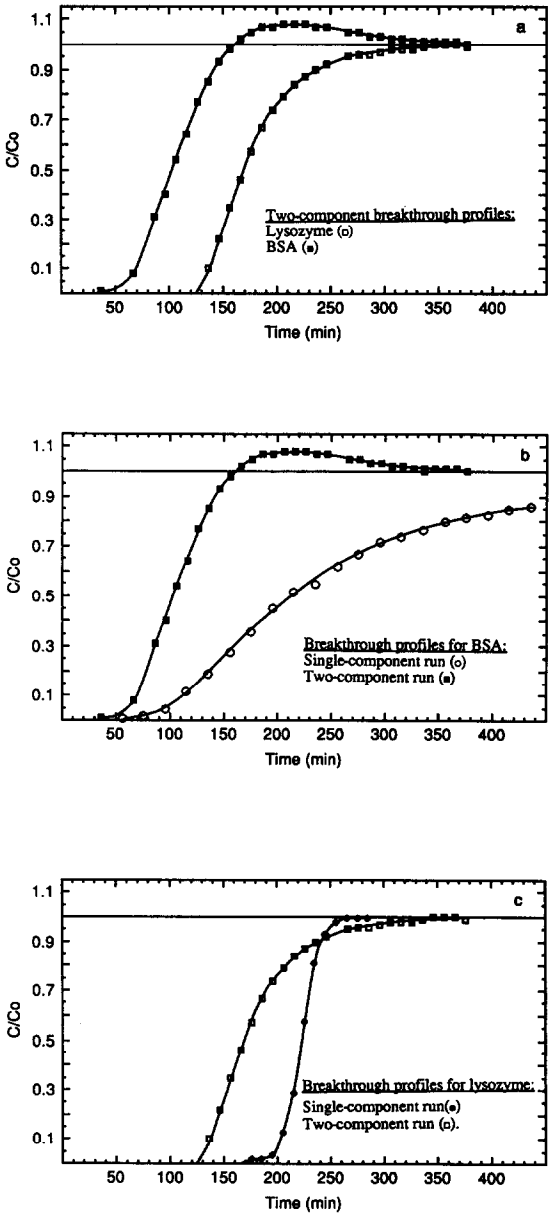

Fig. 2. Breakthrough profiles for the adsorption of lysozyme and BSA to S Sepharose FF in packed beds. The beds used were 1 cm diameter and 2.2 cm high and were loaded at a flow-rate of 1 ml/min in an upward direction. (a) The breakthrough profiles of lysozyme (□) and BSA (■) when a solution containing both proteins, each present at a concentration of 1 mg/ml, was passed through the bed. (b) Breakthrough curves for BSA from experiments in which pure BSA, at a concentration of 1 mg/ml, was applied to a bed (○) and when the mixture of BSA and lysozyme was applied (■). (c) Breakthrough curves for lysozyme from experiments in which pure lysozyme, at a concentration of 1 mg/ml, was applied to a bed (●) and when a mixture of BSA and lysozyme was applied (□).

profile indicates that lysozyme is able to displace, and thereby elute, a certain amount of adsorbed BSA. The amounts of each protein that had bound to the beds were determined by mass balance from the breakthrough profiles. This was performed by integrating the areas above the breakthrough curves for each component taking any area above the $c/c_0 = 1$ line as negative as this represents material eluted from the bed by the more strongly adsorbing component. Allowance was made during these calculations for the void volume of liquid within the bed. It is also possible to use the two models of multicomponent adsorption to calculate the amounts of protein that would be expected to adsorb to the ion exchanger, since at equilibrium the values of c_{01} and c_{02} may be substituted for c_1^* and c_2^* in eqns. 2, 8 and 9. The amounts of each protein bound to the packed bed from experiments and the calculated values from the two models of adsorption are shown in Table II. It can be seen that the experimentally determined figure of 46 mg of BSA adsorbed per ml S Sepharose FF was not accurately predicted by either model but is closer to the value predicted by the fully competitive approach. In the case of lysozyme the experimentally determined figure of 100 mg/ml is almost completely consistent with the fully competitive model.

In order to compare more easily the two-component breakthrough profiles with those obtained from the single-component experiments for each protein, the profiles of multicomponent and single-component experiments have been plotted in the same figures, those for BSA in Fig. 2b and those for lysozyme in Fig. 2c. The single-component breakthrough curves are those determined previously in beds of the same size, under identical conditions and presented in our earlier paper[5]. Fig. 2b clearly shows that the breakthrough profile of BSA in the presence of lysozyme is shifted considerably towards the origin compared to the position of the breakthrough curve when pure BSA is applied to the column. This is a reflection of the fact that significantly more BSA was able to bind to the packed bed in the absence of lysozyme than in the multicomponent experiment. The amount of BSA that was calculated to have adsorbed in the single-component experiment was 130 mg of BSA per ml of S Sepharose FF compared to the 46 mg BSA per ml S Sepharose FF that was bound in the two-component experiment. The slope of the two-component BSA breakthrough profile is seen to be much sharper than that observed in the single-component experiment.

The breakthrough profile of lysozyme obtained in the two-component experiments is shifted towards the origin by a much smaller amount than was the case for

TABLE II

AMOUNTS OF BSA AND LYSOZYME BOUND TO A PACKED BED OF S SEPHAROSE FF AT EQUILIBRIUM

Experimental values were determined using a mixture containing each of the proteins at a concentration of 1 mg mg^{-1} and corresponding figures were calculated from the non-competitive and fully competitive models of adsorption described in the text. All figures are quoted as mg protein adsorbed per ml of S Sepharose FF.

	Experimental (*mg* ml^{-1})	*Non-competitive model* (*mg* ml^{-1})	*Fully competitive model* (*mg* ml^{-1})
BSA	46	100	14
Lysozyme	100	118	103

BSA (Fig. 2c). The position of the two-component curve indicates that although less lysozyme bound to S Sepharose FF in the presence of BSA than was the case when lysozyme alone was present, the difference between the two-component and single-component experiments is not as great as that for BSA, 100 mg of lysozyme per ml S'Sepharose FF having been bound in the two-component experiment compared to an adsorbed lysozyme concentration of 125 mg/ml in the single-component experiment. Also in contrast to the result for BSA, the gradient of the two-component lysozyme breakthrough curve is shallower than that obtained in the respective single-component experiment.

The adsorbed protein profile in packed beds

The development of the adsorbed protein profiles of BSA and lysozyme actually within the packed beds is shown in Fig. 3. The diagrams shows the concentration of

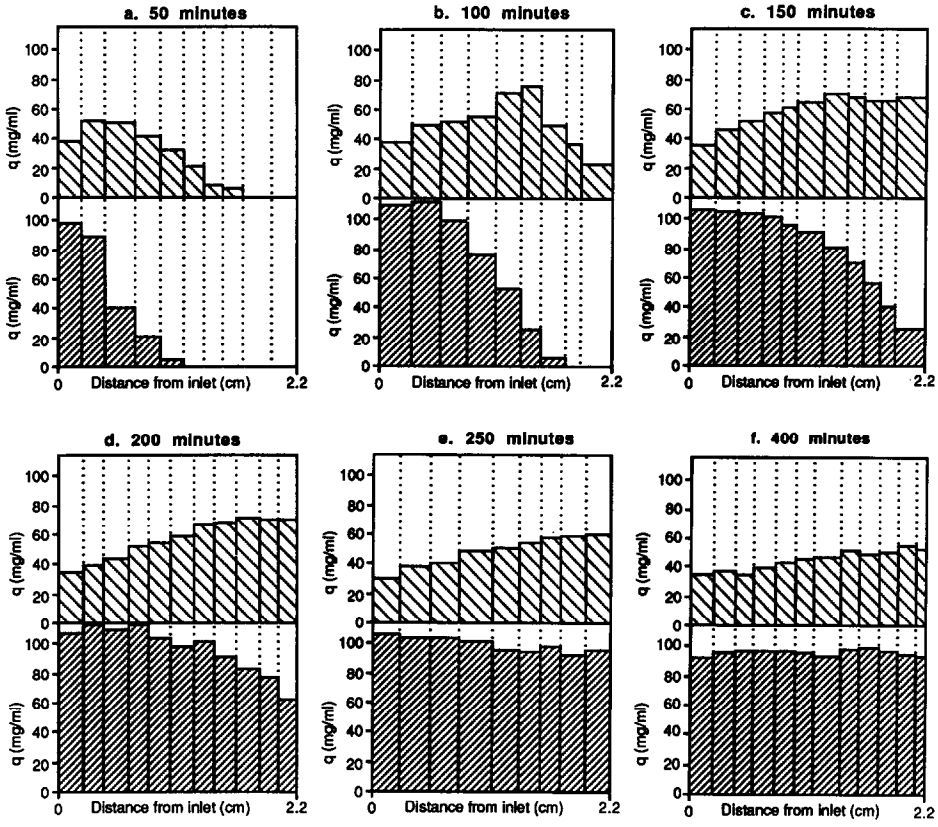

Fig. 3. Adsorbed protein profiles for BSA and lysozyme within packed beds. The beds used were 1 cm diameter and 2.2 cm high and were loaded at 1 ml/min with a solution containing both proteins at a concentration of 1 mg/ml. The BSA profiles are shown in the upper section of each figure in light shading. The lysozyme profiles are shown in the lower section of each figure in dark shading. The results are plotted as the amount of protein adsorbed per ml of S Sepharose FF in each slice against the distance of each slice from the column inlet. The amount of S Sepharose FF in each slice is represented by the different widths of each slice plotted. Each figure shows the profile observed after loading the beds for the following times: (a) 50 min; (b) 100 min; (c) 150 min; (d) 200 min; (e) 250 min; (f) 400 min.

BSA and lysozyme adsorbed on each slice of S Sepharose FF that was taken from a packed bed. The width of each bar in the diagram is proportional to the width of the slices taken from the beds. BSA can be seen to have penetrated further into the bed than lysozyme in the experiments where the column was loaded for 50 and 100 min. This was the expected profile as the breakthrough curves show that BSA appeared in the outlet stream before lysozyme was detected. The adsorbed BSA profile developed a peak which was observed at a maximum in the 100-min experiment. The adsorbed concentration of BSA at this point was 75 mg/ml, far in excess of any previously observed adsorbed BSA concentration in the presence of lysozyme. After this time the peak broadened and developed into a plateau, the concentration of BSA adsorbed on the bed falling as the system approached equilibrium, with the slices closest to the column entry nearing equilibrium first. In the final experiment, in which the column was loaded for 400 min, the amounts of lysozyme and BSA adsorbed, when averaged over the whole bed, were entirely consistent with the values obtained from analysis of the breakthrough curves. The adsorbed concentration of BSA, averaged across the whole bed was 45 mg/ml and the value for lysozyme 96 mg/ml. The figures determined from breakthrough profiles were 46 and 100 mg/ml respectively.

Consecutive application of single-protein solutions to packed beds of S Sepharose FF

Two experiments were performed in which a packed bed of S Sepharose FF was loaded with one protein and then the inlet stream switched to a pure feed of the other protein. The results of these experiments are shown in Fig. 4. The protein profiles are plotted from the point at which the second feed solution first entered the bed. The amounts of protein bound or eluted after the feed was switched were determined from the concentration profiles shown in Fig. 4. In the case of loading a bed with BSA and switching the feed to lysozyme, (Fig. 4a), the concentration of BSA in the exit stream rapidly fell to a low level which during the subsequent FPLC analysis resulted in a peak height that was below the detection limit when the integrator was set at the sensitivity required for lysozyme quantitation. From Fig. 4a it was calculated that approximately 130 mg of lysozyme, equivalent to 74 mg lysozyme per ml S Sepharose FF, bound to a bed which initially contained over 220 mg of adsorbed BSA (125 mg/ml) and of which only 50 mg (29 mg/ml) of BSA was eluted from the column. The result of the complementary experiment in which a bed was loaded with lysozyme and then the feed was switched to BSA is shown in Fig. 4b. In this case the amount of BSA that was adsorbed to the ion exchanger, 29 mg, equivalent to only 17 mg BSA per ml S Sepharose FF, was less than the amount of lysozyme eluted, 40 mg (23 mg/ml).

DISCUSSION

The equilibrium adsorption characteristics of the two proteins, BSA and lysozyme, adsorbing to S Sepharose FF were determined from the single-component adsorption isotherms described previously[5]. The Langmuir isotherm parameters determined are presented in Table I. The equilibrium capacity (q_m) of S Sepharose FF for each protein was found to be similar on a mass basis although due to the greater relative molecular mass of BSA this protein has a smaller q_m value than lysozyme in molar terms. The dissociation constant, K_d, is a measure of the strength of the interaction between the protein and the ion exchanger. In the case of BSA and

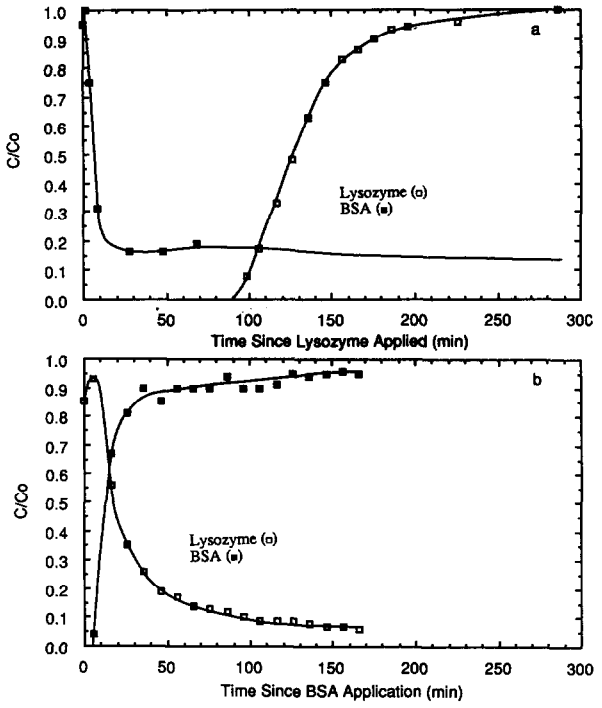

Fig. 4. Adsorption of proteins to packed beds already loaded with another protein. In each experiment the bed was initially loaded with a solution of one protein at a concentration of 1 mg/ml until near to equilibrium with the feed stream. The feed stream was then switched to a solution containing the other protein at a concentration of 1 mg/ml. Concentrations of the two proteins in the exit stream were determined by FPLC analysis and are plotted from the time the feed stream was switched. (a) The profile of BSA (■) and lysozyme (□) in the exit stream of a bed initially loaded with BSA and subsequently loaded with lysozyme. (b) The profile of BSA (■) and lysozyme (□) in the exit stream of a bed initially loaded with lysozyme and subsequently loaded with BSA.

lysozyme adsorbing as single components to S Sepharose FF, lysozyme has a smaller dissociation constant than BSA on both a mass basis and, although the difference is less pronounced, in molar terms. This is probably a reflection of the greater positive charge density found on the lysozyme molecule at pH 5.0. From a consideration of the Langmuir isotherm parameters and the molar concentrations of lysozyme and BSA used in these studies, it was expected that if competitive adsorption occurred, lysozyme would act as the more strongly binding component, with more lysozyme than BSA adsorbing to S Sepharose FF and possibly lysozyme eluting some adsorbed BSA. Such an analysis is supported by predictions from the fully competitive model, using the values of K_{di} and q_{mi} from the single-component studies.

The results of both the two-component batch adsorption and packed-bed experiments did show that greater amounts of lysozyme bound than BSA. However, neither of the models of multicomponent adsorption which were considered correctly predicted the amounts of each protein that bound in either agitated-tank or packed-bed experiments. Since the nature of the adsorption mechanisms of different proteins to an ion exchanger is considered to be based on interactions with the same

charged groups on the ion exchanger surface, it was expected that the experimental results would be in close agreement with the fully competitive model. The major reasons for discrepancies between the observed results and those predicted by the fully competitive Langmuir adsorption model are summarised in Table III and discussed in greater detail below.

In the batch adsorption studies, only the adsorption of BSA was similar to the results of the fully competitive model, with this model greatly underpredicting the amounts of lysozyme that were bound. In contrast, the non-competitive model gave predictions which agreed closely with the amounts of lysozyme that were bound in these batch adsorption experiments, whilst overpredicting the amount of bound BSA. It should be remembered that the experimental results presented in this paper do not constitute a set of competitive adsorption isotherms but rather a collection of paired lysozyme and BSA points, each point lying on a different isotherm. In order to produce a competitive isotherm it is necessary to hold the soluble concentration of one component constant whilst that of the other is varied. Such conditions are impossible to develop in a closed batch adsorption system such as that employed in these studies but can be successfully used in column experiments in which the adsorbent is continually presented with fresh adsorbate solution[3]. Experiments of this kind were not performed in this study due to the length of time (over 5 h) required to allow equilibrium to be established in each column run and in order to construct a set of isotherms many of these experiments are required. Such an approach is therefore

TABLE III

POSSIBLE REASONS FOR THE DIFFERENCES BETWEEN THE OBSERVED EXPERIMENTAL RESULTS AND THOSE PREDICTED BY THE FULLY COMPETITIVE LANGMUIR ADSORPTION ISOTHERM MODEL FOR THE MULTICOMPONENT ADSORPTION OF BSA AND LYSOZYME TO S SEPHAROSE FF

Reason	*Explanation*
Protein size	A large size difference between adsorbing molecules may be reflected in different proportions of the adsorbent surface being available for adsorption with each protein. The fully competitive model assumes that all adsorption sites are available for the adsorption of each molecule.
Protein–protein interactions	If protein–protein interactions occur within the packed beds which are different to those in batch systems as a result of different concentration/time histories of these two systems, more protein may adsorb within the packed bed than is predicted by a Langmuir adsorption model.
Non-equilibrium	The low values of the Langmuir dissociation constants for protein adsorption to ion exchangers suggest that the desorption reactions occur much slower than the adsorption reactions. This may be a reflection of the fact that although protein adsorption occurs via multivalent attachment, only one, or a small number of bonds, may be required to initiate adsorption of a protein whilst all (possibly 10–15) bonds may be required to be broken to desorb a molecule. Consequently it is possible that whilst it was not possible to detect any changes in the soluble protein concentration, protein desorption was still occurring slowly and the results presented do not therefore represent the equilibrium state predicted by the fully competitive model.
Thermodynamics	It is well established that protein adsorption to ion exchangers does not strictly adhere to the thermodynamic assumptions of the single component Langmuir adsorption isotherm. In addition, in the case of multicomponent protein adsorption, violation of the Gibbs–Duhem relationship should also be considered.

better suited to a medium- or high-pressure system such as the non-protein HPLC system used by Jacobson *et al.*[3].

The two extreme models of multicomponent adsorption were also used to calculate the amounts of each protein that would be expected to bind to a packed bed of ion exchanger at equilibrium. In this case the results of the fully competitive model gave a good correlation with the amounts of lysozyme that were bound but the amount of BSA bound was underestimated. These results however, were closer to the fully competitive model than the predictions of the non-competitive model, which overpredicted the amounts of both proteins which should bind, with the discrepancy between predicted and observed results being greatest in the case of BSA.

Despite the lack of exact agreement between the experimental results and those calculated from the fully competitive model, the results of the packed-bed experiments provided substantial evidence, in the form of breakthrough profiles, that some degree of competitive adsorption was occurring. The breakthrough profile of lysozyme was noticeably less sharp in experiments in which a mixture of the two proteins was applied to a packed bed than was the case in single-component experiments. This suggests that the adsorption of lysozyme was hindered by the presence of BSA. In contrast the breakthrough profile of BSA from two-component experiments was sharper than that observed when pure BSA was applied to a bed of S Sepharose FF. The two-component BSA profile was seen to rise above a c/c_0 value of 1, that is the concentration of BSA in the exit stream was greater than that in the inlet stream. This type of profile is caused by a proportion of the more weakly binding component, in this case BSA, being eluted from the adsorbent by the more strongly binding component, in this case lysozyme. Elution of BSA by lysozyme also results in the sharper BSA breakthrough profile observed in the two-component experiments. This effect is analogous to the sharp peak front observed during gradient elution of adsorbates from packed beds. The two-component breakthrough profiles observed are similar to those seen in studies of the multicomponent adsorption of smaller molecules in which competition between adsorbate molecules was occurring[15].

Elution of BSA by lysozyme was also observed in the experiments in which partially loaded beds of S Sepharose FF were extracted and the profile of adsorbed protein determined. In those experiments the amount of BSA adsorbed to the ion-exchange bed passed through a maximum of approximately 80 mg per ml S Sepharose FF after 100 min of loading. At later time points the profile developed into a plateau and after 400 min the amount of BSA on each slice was approximately 40 mg per ml S Sepharose FF. During the experiments lysozyme was seen to penetrate along the length of the bed at a slower rate than BSA, the expected profile as the point of lysozyme breakthrough in the column exit stream is after that of BSA. It is apparent from this profile that the nature of the adsorption process varies along the length of the bed and with time, since in the early stages, at the regions of the bed furthest from the column inlet there is no lysozyme present in the soluble phase and BSA is absorbing as a single component and hence would be able to bind at all sites with no competition occurring. At the column inlet and, as time progresses, over a greater length of the column, adsorption of both proteins occurs simultaneously.

In order to investigate the characteristics of adsorption of protein to ion exchanger which is already loaded with another protein, the experiments in which packed beds of S Sepharose FF were first loaded with one protein and then the feed

solution switched to a solution of the other protein were performed. From consideration of a competitive model of adsorption, supported by the evidence of the breakthrough profiles, it was expected that lysozyme would elute and replace a proportion of the adsorbed BSA. Conversely it was expected that BSA, the less strongly binding component, would be less effective at replacing adsorbed lysozyme. Similar experiments of protein adsorption/displacement in a reversed-phase liquid chromatography system[16] showed just such behaviour. However in this study, the results of applying lysozyme to a bed near to saturation with BSA were found not to correspond to this expectation. The amount of BSA which was eluted from the bed was approximately 30 mg per ml S Sepharose FF, with the final adsorbed concentration of BSA being just under 100 mg per ml S Sepharose FF, whilst an additional 75 mg lysozyme was bound per ml S Sepharose FF. These results are in contrast to those reported by Di Bussolo and Gant[16], who demonstrated complete elution of more weakly binding proteins by more strongly adsorbing proteins. However it should be noted that in that system, significant concentrations of the reversed-phase liquid chromatographic elution agent, 20% acetonitrile, were present in the equilibration buffer. A comparable experiment in the ion-exchange system used in this study would therefore include the presence of salt in the buffers (which in this case had a relatively low ionic strength), in order to weaken the strengths of interaction of both proteins. In the complementary experiment, in which BSA was applied to a bed saturated with lysozyme, as expected the more weakly binding BSA was found to be less effective at eluting and replacing adsorbed lysozyme.

The results of the experiments described have clearly shown that the adsorption of BSA and lysozyme to the ion exchanger S Sepharose FF is, to some extent, competitive in nature. However, the discrepancies between the experimental results and those predicted by either model of multicomponent adsorption indicate that the adsorption process is more complex than the models described. One of the primary assumptions of the fully competitive model is that all adsorption sites are equally accessible to all adsorbate molecules. However it is likely that due to the smaller size of lysozyme in comparison to BSA, lysozyme is able to penetrate regions of the ion exchanger particles which are too restricted for BSA to enter. Such differential access of proteins of different molecular sizes to adsorbent particles is the principle on which molecular-exclusion chromatography is based and underivatised Sepharose is marketed as a gel filtration material. Any lysozyme adsorption which occurred at sites which are inaccessible to BSA would be non-competitive and would result in the amount of adsorbed lysozyme being underpredicted by the fully competitive model. The presence of large quantities of BSA adsorbed within the particles might be expected to hinder the access of lysozyme to these sites.

It should be remembered that predictions from the fully competitive model refer to the amounts of protein that would be bound to the ion exchanger at equilibrium with the liquid phase. It is possible that equilibrium may not have been achieved in some of the adsorption experiments described here and this may be a further reason for the discrepancies between the predictions and the experimental results. Although the lack of variation of the concentrations of proteins in the liquid phase as measured by FPLC appeared to suggest that the systems were at equilibrium, adsorption/desorption may have been still occurring at slow rates. The mechanism for the displacement of an adsorbed molecule by another is unknown but it is possible that an adsorbed

molecule may have to desorb from the ion exchanger as a result of the reversible nature of the interaction before another molecule can be bound. Since it is likely that proteins adsorb to ion exchangers by multivalent attachment[2] then desorption of a protein molecule requires that many bonds be broken before a protein molecule becomes detached from an adsorbent surface. In the case of BSA and lysozyme, the dissociation constants of the protein–adsorbent interaction are small, suggesting that the desorption rates may indeed be slow.

A further adsorption mechanism could be due to protein–protein interactions within the ion exchanger particles. Electrostatic interactions between lysozyme and BSA in free solution have been reported previously[17,18]. Although such interactions were not reported at pH 5, the pH used in these adsorption studies, the large amounts of protein adsorbed within the ion exchanger particles and the possibility that they are adsorbed in specific orientations, may promote such interactions, with the result that protein is adsorbed within the ion exchanger but to other protein molecules rather than ion exchanger functional groups. The BSA molecule has an ellipsoidal shape and the charge distribution along the major axis is known to be asymmetric, such that one end of the molecule carries predominantly positively charged groups and the other end negatively charged groups. Studies of BSA adsorption to a silica-based ion exchanger in which the available surface area was known have indicated that BSA molecules were adsorbed in an "end-on" orientation, that is with the major axes perpendicular to the adsorbent surface[19]. Adsorption of BSA to lysozyme in such an orientation with the positively charged region of the molecule interacting with the negatively charged sulphonic groups on the surface of the ion exchanger, would present the negatively charged region of the BSA molecule pointing out towards the bulk liquid. This may have provided sites with which the strongly positively charged lysozyme molecules could have interacted electrostatically, resulting in adsorption. Any lysozyme adsorbed in this manner would be to sites which would not be available to BSA and would be in addition to those available during single-component adsorption of lysozyme. Under these circumstances the amount of lysozyme predicted to adsorb by the fully competitive model would indeed be an underestimate of the experimental results. It should be emphasised that we are not postulating that such an adsorption mechanism is a general phenomenon but one that may be pertinent to the system under study in this paper. However it should always be borne in mind that at the high protein concentrations found within packed beds and the likelihood of adsorption imposing some kind of order on the orientation of adsorbed protein molecules, then interactions which are not normally seen in free solution may occur within a packed bed.

CONCLUSIONS

The studies presented here have demonstrated some of the experimental techniques and a possible theoretical approach that can be used to investigate multicomponent protein adsorption. In this study multicomponent adsorption isotherms were not determined experimentally and in future studies it would be instructive to determine such isotherms by performing frontal analysis experiments using solutions containing mixtures of varying compositions[3].

Two extreme models of multicomponent protein adsorption have been considered and neither of these accurately predicted the adsorption characteristics of BSA

and lysozyme in either batch adsorption or packed-bed experiments. Clear evidence that a competitive model is the better approach to modelling multicomponent protein adsorption to ion exchangers was provided by the breakthrough curves and the profiles of protein adsorbed along the length of packed beds. Competitive adsorption was demonstrated in those experiments by the observation that lysozyme could elute adsorbed BSA from the ion exchanger. However the discrepancies between the observed and predicted results suggest that in studies of the multicomponent adsorption of proteins of different sizes, it may be necessary to include in the model contributions from non-competitive adsorption in order to allow for the adsorption of small proteins in regions of particles inaccessible to larger molecules. In the particular case of BSA and lysozyme, the development of such a multicomponent adsorption model is yet further complicated by the possibility that lysozyme may become bound to adsorbed BSA molecules as a result of electrostatic interactions. In addition, the suitability of BSA generally as a model protein should be seriously questioned, as its behaviour as a single component can be complicated by interactions with itself and the consequent formation of dimers in the packed bed[5].

In conclusion, the results presented here suggest that the theoretical modelling of multicomponent protein adsorption is a complicated task and that an accurate model may require contributions from the theories of molecular-exclusion chromatography and protein–protein interaction in addition to adsorption chromatography.

ACKNOWLEDGEMENTS

The authors would like to thank the Science and Engineering Research Council for financial support. They are also grateful to Pharmacia LKB Biotechnology (Uppsala, Sweden) for the provision of experimental materials and equipment and to Brenda Horstmann for constructive comments on the text.

SYMBOLS

c	liquid phase concentration of protein
c_0	initial or inlet liquid phase protein concentration
K_d	dissociation constant for the protein–ion exchanger complex
q	concentration of protein adsorbed to the ion exchanger
q_m	maximum protein capacity of the ion exchanger
v	volume of ion exchanger
V	volume of liquid
θ	fractional occupancy of adsorption sites with protein at equilibrium
*	value when system is at equilibrium

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